EXHIBIT J

Drugs of Today 2000, 36(2-3): 155-162

CCC: 0025-7656/2000

SIGNIFICANCE OF THE NITRIC OXIDE CASCADE IN ERECTILE FUNCTION

Arthur Burnett

Johns Hopkins Hospital, Baltimore, Maryland, USA

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Summary

In recent years, a significant body of experimental evidence has accumulated implicating nitric oxide as the central component of a novel signal transduction system that functions in the penis to mediate penile erection. The involvement of nitric oxide in erection physiology appears to be quite complex, involving multiple regulatory interactions: this gaseous molecule has been found to trigger several biochemical events that elicit erectile tissue responses, while a host of modulatory factors have been identified that influence its release and action in erectile tissue. Ongoing investigations in nitric oxide biology in other organ systems also suggest mechanisms which, while yet to be fully established in the penis, may operate significantly to determine the role of nitric oxide in this organ. Further elucidation of cellular and molecular interactions involving nitric oxide effects in the penis can be expected to reveal diverse targets that may serve as a basis for novel pharmacotherapeutic strategies for the future management of erectile dysfunction.

Correspondence: Arthur L. Burnett, M.D., Marburg 407, Johns Hopkins Hospital, 600 North Wolfe Street, Baltimore, MD 21287, USA.

Introduction

The recent discovery that the nitric oxide/3', 5'-cyclic guanosine monophosphate (cGMP) signal transduction cascade is the premier biochemical mechanism responsible for penile erection has been rapidly translated into the clinical development of the first effective oral pharmacotherapy for erectile dysfunction, sildenafil citrate (Viagra®). This medication works precisely to augment the erectile response, elicited by nitric oxide stimulation of cGMP production, by inhibiting specifically type V phosphodiesterase catalysis of cGMP to its inactive form (1). As a result, cGMP remains in abundance to promote the biochemical cascade leading to the relaxation of penile vascular and corpus cavernosal smooth cells, a critical biological step in the erectile response (2).

This therapeutic development might suggest that the nitric oxide-based biochemical signalling pathway has been amply exploited to treat erectile dysfunction. However, such notion is probably mistaken. From a biochemical perspective, sildenafil does not represent a primary effector of the nitric

oxide-mediated erectile response since its influence apparently requires the front-end formation of nitric oxide and its subsequent stimulation of cGMP production in the penis. From a clinical perspective, it has been observed that this medication does not satisfactorily treat erectile function in all men, and in fact in clinical usage the medication yields an approximate 70% response rate for all varieties of erectile dysfunction (3). This response rate diminishes to the range of 40% in men using the medication following radical prostatectomy, an outcome which has been explained by the likely surgical injury to cavernous nerves that principally liberate nitric oxide into the erectile tissue (4). These basic research and clinical observations indicate that the fundamental components of the biochemical process producing erection involving the formation, release and action of nitric oxide remain areas of critical scientific interest.

Scientific investigations are ongoing that center on elucidating the cellular and molecular mechanisms pertaining to nitric oxide biology in the penis that may be targeted for pharmacological manipulation of the erectile response. It is reasonable to conjecture that some manner(s) of precise regulatory control operates to determine the effects of nitric oxide in the penis, much like mediators acting in other organs and organ systems which are neither released tonically or unalterably but are precisely released and modified by various modulatory factors. Potential modulators of nitric oxide effects in the penis include locally active neurotransmitters, hormones, paracrine factors, and even exogenously administered substances. Regulation may also involve the molecular mechanisms involved in the gene expression of the enzyme pathways responsible for nitric oxide synthesis, which may in fact occur in a genitourinary tissue-specific manner. Given its multiple actions, the molecule could conceivably affect the contractility of corpus cavernosal smooth muscle tissue in ways that do not directly involve cGMP production or function, and its biochemical pathway may involve coordination with other intracellular signalling pathways. These possibilities all support the notion that an elaborate nitric oxide regulatory system operates in the penis which would seemingly exert profound effects on the local message delivered by this molecule. Such a regulatory system may have more importance with regard to the nitric oxide-mediated effects in this organ than the biological action of the chemical factor alone.

The purpose of this review is to provide a contemporary perspective regarding the biology of nitric oxide in the penis, referable to its well-supported, significant role in the physiology of penile erection. The following discussion begins with a brief overview of the cellular basis for nitric oxide function in the penis and then proceeds with a summary of scientific work that pertains to diverse biochemical and molecular regulatory interactions associated with nitric oxide effects in the penis. Accordingly, further insight into the mechanisms governing the functional role of this chemical in the penis may promote the development of new techniques for controlling its release in this organ with clinical relevance to the treatment of erectile dysfunction.

Nitric Oxide Biosynthesis and Function in the Penis

The significance of nitric oxide in the mediation of penile erection parallels the profound biological role that has been demonstrated for this messenger molecule across multiple scientific disciplines over the last decade. While nitric oxide is indeed a free radical, evanescent gas, it exerts biological roles as a vasodilator, neurotransmitter, antimicrobial effector, and immunomodulator (5, 6). Key biochemical properties of nitric oxide relate to its sources and mode of synthesis and its novel use of signal transduction (defined as the intracellular or transcellular delivery mechanism whereby an initially released chemical signal is transmitted and amplified by a second messenger molecule) (Fig. 1).

Nitric oxide is derived from the terminal guanidino group of L-arginine, its substrate, under the catalytic activity of nitric oxide synthase (NOS), with equimolar formation of L-citrulline as a by-product. The NOS enzyme refers to a family of three distinct NOS isoforms, conventionally understood to represent the products of three different genes. Two NOS isoforms are continuously present and have thereby been termed constitutive NOS. Their identification as neuronal and endothelial types pertains to the cells of origin to which they were initially localized. Both constitutive NOS isoforms require calmodulin and reduced nicotinamide adenine dinucleotide phosphate (NADPH) for catalytic activity and generate nitric oxide transiently in low amounts, appropriate for cell-cell signaling, following a rise in intracellular calcium and calcium-calmodulin binding. The inducible NOS isoform by comparison exists primarily in macrophages, although it may be expressed in other cells particularly after cytokine (immune

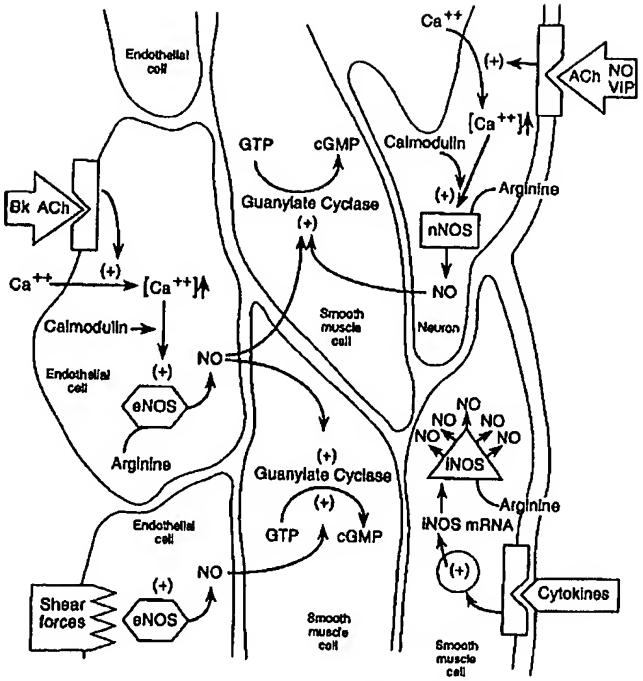

Fig. 1. Proposed mechanisms for nitric oxide (NO) synthesis, action and regulation in penis. Nitric oxide is constitutively produced from its precursor, L-arginine, in endothelial cells and neurons by catalytic action of the endothelial and neuronal isoforms of nitric oxide synthase, eNOS and nNOS, respectively. Whereas messenger molecules commonly activate these enzymes by signaling influx of calcium and its binding with calmodulin, other biochemical or mechanical factors may interact with this process controlling the formation of nitric oxide. Once synthesized, nitric oxide diffuses to local smooth muscle cells where its primary mode of action is to bind with and activate guanylate cyclase that converts 5'-guanosine triphosphate (GTP) to 3',5'-cyclic guanosine monophosphate (cGMP). Smooth muscle cells represent another potential source of nitric oxide but they appear to require cytokine stimulation of inducible NOS (iNOS) expression. Bk: bradykinin; ACh: acetylcholine; VIP: vasoactive intestinal peptide. (Reproduced with permission from Burnett, A.L. *Nitric oxide in the penis: Physiology and pathology.* J Urol 1997, 157: 320-324.)

response modulator) induction, and releases nitric oxide in a calcium-independent fashion usually in large, noxious amounts. The variable release of nitric oxide, depending on site and magnitude, appropriately determines the physiologic or pathophysiologic outcome of its release.

Support for nitric oxide as the principal mediator of penile erection has derived from various biochemical, histochemical, and physiologic studies (7, 8). *In vitro* organ bath studies demonstrating nitric oxide-mediated relaxant effects initially in isolated rodent anococcygeus and bovine retractor penile muscles and subsequently in human and animal corpus cavernosal tissue specimens provided early evidence for this role. A common strategy among these investigations was the application of a nitric oxide donor or the NOS substrate, L-arginine, to elicit tissue relaxation, which resembled electrical (neurogenic) stimulatory effects, and conversely the administration of agents that block nitric oxide synthesis or its mode of action in order to abolish relaxant effects. *In vivo* animal paradigms of penile erection were also studied using similar methodologies, thereby affirming the regulatory role of nitric oxide in physiologic penile erection. Biochemical measurements of NOS activity and localizations of NOS by immunoblot analysis and immunohistochemistry in

human and animal genitourinary tract structures using neuronal NOS-specific antibodies have strengthened the scientific basis for NOS and nitric oxide mediation of erectile function in the penis.

Presently, a preponderance of evidence supports the concept that nitric oxide derives from the autonomic innervation of the penis and operates as a neurotransmitter of nonadrenergic, noncholinergic-mediated penile erection. Neuronal NOS is believed to be the primary NOS isoform which constitutively releases this neurotransmitter, in accordance with its localizations to nerves projecting from local pelvic ganglia and terminating within the erectile tissue of the penis as well as to neuronal circuitry at the spinal cord level influencing function of the peripheral organ. Endothelial NOS has been localized to vascular and trabecular endothelium within the penis and is perceived to provide an auxiliary source of nitric oxide for the production of penile erection. Inducible NOS expression has been identified in corpus cavernosal smooth muscle cells indicating that this cellular source may contribute to the local availability of nitric oxide in the penis, although the inducible NOS catalysis of nitric oxide production requires cytokine stimulatory conditions (9). On this basis, and in light of additional localization studies that have not confirmed the normal presence of inducible NOS in normal erectile tissue (10), the inducible NOS isoform would appear unlikely to discharge momentary physiologic activities of the penis.

The mechanism of action of nitric oxide that yields corpus cavernosal smooth muscle relaxation responsible for penile erection, similar to nitric oxide-mediated smooth muscle relaxant responses elicited in vasculature and the gastrointestinal tract, is associated with a novel form of signal transduction. Among a host of biochemical pathways that carry out functions initiated by nitric oxide, the most prominently characterized pathway involves nitric oxide-stimulated production of cellular cGMP, which then works as a second messenger molecule. Following its production and release, nitric oxide diffuses locally into adjacent cells and binds with intracellular guanylate cyclase, serving as a physiologic "receptor" for the molecule, which thereby induces a conformational change in this enzyme and activates it. Once activated, guanylate cyclase catalyzes the conversion of 5'-guanosine triphosphate to cGMP.

Cyclic GMP then proceeds to activate specific protein kinases that alter the contractile state of smooth muscle through varied possible mechanisms, including direct dephosphorylation of myosin light chain cross-bridging, control of calcium and potassium ion fluxes and stores, and interaction with other signal transduction mechanisms (7, 11). Recent experimental studies also indicate that an alternative mechanism for the cGMP effect in corpus cavernosal smooth muscle involves interference with myosin light chain cross-bridging unassociated with dephosphorylation (12). Alternative cGMP-independent mechanisms have also been studied as possible ways that nitric oxide mediates corpus cavernosal smooth muscle relaxation. Relaxant effects in human tissue specimens have been shown to involve the stimulation by nitric oxide of Na^+/K^+-ATP, a mechanism which may then cause direct hyperpolarization of the smooth muscle cell or inhibition of calcium influx leading to attenuation of tissue contraction (13). Nitric oxide has also been demonstrated to modulate a potassium conductance pathway operating at the rabbit corpus cavernosal smooth muscle cell membrane level, resulting in the smooth muscle cell hyperpolarization that yields tissue relaxation (14).

Biochemical Regulation of Nitric Oxide Release in the Penis

In view of the biochemical factors required for the standard production and release of nitric oxide, suboptimal conditions such as hypoxia, low intracellular calcium stores and an extreme acid-base milieu have been implicated in hindering the biosynthesis of the molecule (8, 15, 16). The biochemical transformation or metabolism of nitric oxide also influences its potential effects. In biological tissues, nitric oxide has the ability to react with oxyhemoglobin, convert into the metabolites nitrite and nitrate, and react with superoxide anion, all reducing its availability (7). Advanced glycosylation end products directly inactivate nitric oxide by an antiproliferative effect, implicating this biochemical process in the erectile dysfunction associated with diabetes (17). Increasing the availability of the NOS substrate, L-arginine, may provide an approach to promote the production of nitric oxide in the penis. Experimental testing supports this hypothesis since the erectile dysfunction of aging rats has been shown to be ameliorated following long-term oral administration of high doses of L-arginine (18).

The regulatory basis of nitric oxide also pertains to biochemical and even physical

mechanisms that may influence the catalytic basis of NOS function. Nitric oxide itself has been postulated to exert a direct feedback inhibition of NOS activity by interacting with the heme moiety of the enzyme (6, 7). Androgens have been implicated in maintaining nitric oxide-mediated erectile function, with the assumption that the hormonal regulation may in part pertain to interactions with known regulatory sites of the enzyme (7, 8). Interestingly, it has been shown that electrical stimulation of the cavernosal nerve that elicits physiologic penile erection transiently restores depleted penile NOS activity levels in castrated rats to that of intact, nonstimulated animals (19). The activity of NOS can also be influenced by alternative signal transduction systems, which invokes another mechanism that regulates nitric oxide effects in the penis. Both 3', 5'-cyclic adenosine monophosphate (cAMP)-dependent protein kinase and protein kinase C, a product of the phosphoinositide system, can directly phosphorylate NOS and thereby reduce its activity (20).

In keeping with the notion that nitric oxide functions as a neurotransmitter, various neurogenic stimuli have been associated with the physiologic release of nitric oxide. Ample experimental evidence supports the involvement of neuroactive amino acids such as glutamate, acting through presynaptic *N*-methyl-D-aspartate (NMDA) receptor activation, in nitric oxide neurotransmission in the brain (21). Whether this mechanism is important for nitric oxide release in peripheral nerves supplying the penis or other parts of the lower genitourinary tract requires investigation. Co-transmission involving nitric oxide and other neuronal agents has been explored in various organs. In the penis, neuropeptides such as vasoactive intestinal peptide, long viewed as a major ancillary nonadrenergic, noncholinergic neurotransmitter of penile erection, may induce erectile responses through nitric oxide-cGMP biochemical pathway mediation. Vasoactive intestinal peptide co-localizes with NOS in human and rat penile neurons, and vasoactive intestinal peptide-induced relaxation of rabbit corpus cavernosal tissue is attenuated by inhibitors of NOS or guanylate cyclase (22). Similarly, the nitric oxide-cGMP pathway has been shown in physiologic tension experiments of isolated corpus cavernosum from various species to modulate noradrenergically mediated tissue tone (23). The roles of vasoconstrictive endothelial factors such as endothelins which when released oppose (and thus regulate) the relaxant effects of nitric oxide in the penis has also been advanced (24). Notwithstanding neuronally released nitric oxide, the physiologic liberation of the molecule from endothelium requires stimulation. Acetylcholine as well as other factors such as substance P and bradykinin have been proposed to elicit nitric oxide release from endothelial sources in the penis (22).

Recent intense scientific investigation has confirmed the roles of neuronal NOS-associated proteins which influence the neurotransmission of nitric oxide. These proteins govern the physical interactions of neuronal NOS that determine whether the enzyme is functionally active or inactive. The synaptic association of neuronal NOS in neurons is mediated by the binding of the PDZ/GLGF motif, located in the 5'-flanking region of the enzyme, to membrane proteins such as postsynaptic density proteins PSD-95 and/or PSD-93 (25). Other proteins antagonize neuronal NOS function. Protein inhibitor of NOS (PIN) serves to inhibit the function of neuronal NOS by destabilizing its active dimeric conformation (26). This purpose is postulated to be associated with controlling pathologic nitric oxide release in the brain that occurs with several models of neurotoxicity and neuronal cell death. Carboxyl-terminal PDZ ligand of neuronal NOS (CAPON) binds directly with the neuronal NOS PDZ domain, thereby controlling the synaptic membrane neurotransmitter release actions of the enzyme (27). Accordingly, the physiologic significance of this interaction may be to determine the translocation of neuronal NOS intracellularly as a mechanism for achieving neurotransmitter specificity for nitric oxide release.

Molecular Regulation of Nitric Oxide Release in the Penis

The molecular basis for the generation and delivery of nitric oxide release in the penis represents another mode of regulatory control directing the effects of this chemical factor. The molecular research of NOS dates back to 1991 when Bredt, Snyder and colleagues cloned and sequenced the neuronal NOS complementary DNA from rat cerebellum (28). Since that time, neuronal NOS has been cloned from the brain of other species including human and mouse, with the finding that the genes are highly conserved across species (5, 6). The human neuronal NOS gene is localized to chromosome 12, region 12q24.2, is approximately 160 kb in size, and is composed of 29 exons and

28 introns, with translation into its protein product starting from exon 2 and terminating in exon 29.

This basic foundation in the molecular biology of neuronal NOS is important since it allows a critical examination of the potential mechanisms of its regulation at the transcriptional and post-transcriptional levels. Precisely, this understanding may explain various observations regarding neuronal NOS that conveys its necessary functional role. Accordingly, the identification of two distinct promoters in exon 1 of the human neuronal NOS gene that produce alternative splicing of the NOS messenger ribonucleic acid (mRNA) is not expected to affect the actual size of the translated neuronal NOS protein since the promoters are located in the 5'-untranslated region of the gene. A similar situation appears to occur with regard to a dinucleotide microsatellite repeat contained in the 3'-untranslated region in exon 29 of the human neuronal NOS gene, although the significance of this observation relates to the fact that multiple alleles of the repeat exist in normal individuals indicating polymorphism. Various conditions including cerebral ischemia in the rat brain and somatic nerve damage in the rat dorsal root ganglia have been associated with increases in neuronal NOS mRNA levels, although the precise molecular mechanisms responsible for these effects remain unclear. The likely involvement of neurotrophic mechanisms may partly explain these effects (5, 6). Such a hypothesis has been tested in rodent models of cavernosal nerve injury whereby the administration of such factors as somatomedin stimulators (29) and neuroimmunophilins (30; Sezen and Burnett *et al.*, unpublished data) has enhanced the recovery of erectile function concomitant with regeneration of neuronal NOS-containing penile nerves.

The molecular basis of neuronal NOS expression certainly reconciles the predicament that neuronal NOS knockout mice, supposedly lacking nitric oxide production from neuronal sources, are observed to preserve erectile function and reproduce (31). These mice were generated by the targeted deletion of exon 2, under the premise that this genomic disruption should effectively eliminate the initiation region responsible for translating the neuronal NOS protein. Erections were shown to remain NOS-dependent in these animals explained initially on the grounds that the existing and apparently upregulated endothelial NOS isoform expression compensatorily overcomes the neuronal NOS deficiency (10). More recent explanations for the retained erectile function in these mice have turned to the probable role played by neuronal NOS variants resulting from alternative mRNA splicing that are distinct from the primary exon 2-containing neuronal NOS (termed neuronal NOS-α). The identification of actually expressed neuronal NOS variants (termed neuronal NOS-β and neuronal NOS-γ) that begin alternative translation in exon 1 in mice, unlike that occurring in humans in which this region is untranslated, has offered a basis for the persistent neuronal NOS function in select brain regions of the knockout mice (32, 33). Molecular studies applied to the lower genitourinary tract of the knockout mice have now confirmed similar expression of neuronal NOS variants, which could account for the preserved erectile function in these animals (34). These investigations reestablish the importance of nitric oxide, derived from neuronal NOS, in the erectile process.

Alternative neuronal NOS mRNA splicing has emerged as an important basis for the structural diversity of the gene, which may afford mechanisms for tissue-specific neuronal NOS regulation (5, 6, 32). An alternatively spliced isoform of neuronal NOS was initially confirmed to be expressed in human differentiated skeletal muscle (designated neuronal NOS-μ), with a slightly larger molecular size than the neuronal NOS protein expressed in brain because of an alternatively spliced segment between exons 16 and 17 (35). The same neuronal NOS-μ was subsequently cloned from rat penile corpora cavernosa (36). The neuronal NOS-μ transcripts were identified to be uniquely expressed in the rat penis, urethra, prostate, and skeletal muscle, whereas these coexisted with the cerebellar neuronal NOS in the pelvic plexus, bladder, and cerebellum. These research directions have opened the possibility that alternative mRNA splicing may be characterized and exploited as a molecular target in the human to direct genitourinary tissue-specific responses.

The molecular basis for endothelial NOS regulation also pertains to the molecular characterization of this gene including the transcriptional and post-transcriptional determinants of its expression (5, 6). The cloning and sequencing of endothelial NOS from cultured bovine and human endothelial cells have revealed that the human endothelial NOS protein has an approximately 94% homology to the bovine endothelial NOS protein and an ~50% homology to the human neuronal NOS

isoform. The human endothelial NOS protein has been mapped to chromosome 7, region 7q35-36, is approximately 21 kb in size, and is composed of 26 exons and 25 introns. Promoter elements have been identified in the 5'-flanking region, which are conserved in the constitutively expressed protein. Certain growth factors and cytokines, molecular oxygen tension, and local blood flow phenomena are included among those factors thought to exert transcriptional regulation of endothelial NOS expression (5, 6). Blood flow apparently exerts a mechanical effect associated with an endothelial shear response element located on the 5'-flanking promoter region of the endothelial NOS gene. At a post-translational level, diverse protein-protein interactions have been shown to determine the subcellular localization, stability and functional activation of endothelial NOS (5, 6). The possibility that these multiple molecular mechanisms apply to the endothelium within the penis invites further scientific investigation that may yield novel targets for enhancing nitric oxide-mediated erectile function.

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